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Taenia solium cDNA sequence encoding a putative immunodiagnostic antigen for human cysticercosis

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Abstract

A *T. solium* metacestode cDNA library was prepared and antibody screened to obtain recombinant antigens, which could be used for the neurocysticercosis diagnosis. The F18 clone was selected and sequenced, and the full length cDNA characterised as well as the genomic structure from the gene. F18 is a single copy gene that spans ~6.1 kb and contains five exons and four introns. The F18 cDNA has a 690-nucleotide open reading frame that encodes a putative polypeptide of 229 amino acids with a predicted molecular mass of 26.06×10^3 M_r. The F18 recombinant protein was obtained and purified by affinity chromatography using pGEX system (G-F18) and pQE system (H-F18). The purified G-F18 fusion protein showed the best results when it was used in ELISA with sera from neurocysticercosis patients.

Keywords: *Taenia solium*; Genomic structure; Cysticercosis; Recombinant proteins; cDNA

1. Introduction

Taenia solium and *Taenia saginata* are two taeniids of great economic and medical importance, causing bovine and porcine cysticercosis and taeniasis in humans. In addition, *T. solium* eggs can infect humans, then the *T. solium* metacestode may also develop in humans, producing cysticercosis, with neurocysticercosis (NCC) occurring as a frequent and sometimes fatal complication [1]. Moreover, the disease is currently recognized as the main cause of acquired epilepsy in the world (COMMISSION ON TROPICAL DISEASES, 1994). Infections with these cestodes are therefore a serious health problem in areas of endemicity such as Latin America, Asia and Africa [2,3]. In addition, an increase in the number of cases in areas of non-endemicity has been observed in recent years [4].

Diagnosis of *T. solium* cysticercosis of humans is an unresolved problem. Therefore, the development of a reliable and specific serological diagnosis is urgently needed [5]. The scarcity of parasite material handicaps the development of suitable immunodiagnosis and, in addition, the crude parasite extracts that may be employed for diagnosis are non-specific [6,7]. The enzyme-linked immunoelectrotransfer blot (EITB) assay [8], based on the detection of specific antibody to defined parasite glycoprotein antigens, provided an improved antibody detection assay. This

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test has a documented specificity near 100% and a sensitivity of 94% to 98% for patients with two or more cysts or enhancing lesions. However, this assay has two important limitations. Firstly, this test is frequently false negative in patients with single intracranial cysticerci, in which fewer than 50% tests are positives [9]. Secondly, the cyst lectin-bound glycoproteins (LLGP) require sophisticated equipment for consistent purification [10]. On the other hand, the diagnosis by computed tomography and magnetic resonance imaging are expensive and inaccessible in most areas where NCC is endemic [11]. However, an expressed recombinant antigen would circumvent such limitations [12,13]. On the other hand, synthetic peptides offer an alternative to recombinant antigens [10]. With this in mind, a *T. solium* metacestodes expression library was prepared for the expression of *Taenia* gene products with relevance for diagnosis. In the present report we describe the cloning and characterization of a *T. solium* recombinant antigen, F18, and examine its diagnostic potential.

2. Experimental

2.1. Parasites

T. solium metacestodes were extracted from naturally infected Mexican pigs with cysticercosis and stored in liquid nitrogen until use. These metacestodes were employed for construction of both an expression cDNA library and a genomic library.

2.2. Rabbit sera

The *T. solium* metacestode cDNA library was screened using a pool of the following sera.

(i) Sera from rabbits immunized with excretion/secretion antigens from *T. saginata* metacestodes.
(ii) Sera from rabbits immunized with cyst fluid from *T. saginata* metacestodes.
(iii) Sera from rabbits immunized with 35, 57 and 95×10^3 M_r proteins from *T. saginata* metacestodes.
(iv) Sera from rabbits immunized with glycoproteins extract from *T. solium* metacestodes.
(v) Sera from rabbits immunized with cyst fluid from *T. solium* metacestodes.

2.3. Human sera

Sera from patients with helminth infections: 22 sera samples of acute NCC patients with lesions highly suggestive of NCC on neuroimaging studies (computed tomography, CT, and/or nuclear magnetic resonance imaging, MRI), and clinical findings characteristic of the disease. Specific antigen detection by ELISA using the monoclonal antibody HP-10 [10] demonstrated that 64% of sera had excretory–secretory metacestode antigens. These sera were supplied by Dr. Dávila from the Parasitology Department of Carabobo University, Valencia, Venezuela. Fifty sera samples from patients: (i) 30 sera from patients, who live in endemic areas of Venezuela, supplied by Dr. Milagros Cortez from the BIOMED of Carabobo University, Maracay, Venezuela, with NCC confirmed by image analysis (CT and/or MRI), and identified as seropositive by ELISA using *T. solium* metacestodes cyst fluid as antigen [14]. (ii) Twenty sera samples from Latin America immigrants, who live in Spain, with lesions highly suggestive of NCC confirmed by image analysis (CT and/or MRI), clinical and epidemiological criteria and identified as seropositive by the RIDASCREEN® *Taenia solium* IgG commercial diagnosis kit (R-Biopharm, Darmstadt, Germany). These sera were supplied by Dr. Mercedes Rodriguez from the Parasitology Department of Instituto de Salud Carlos III, Madrid, Spain.

In addition, 33 sera from individuals with other helminth infections such as hydatidosis ($n=14$) confirmed by image analysis and serological analysis, fasciolasis ($n=9$) and schistosomiasis ($n=10$) confirmed by serological methods, were also examined.

2.4. Negative sera

The negative control population consisted of 35 sera from healthy European individuals, without a history of having visited endemic areas, and were

used to establish suitable cut-off values for the serological assays.

2.5. cDNA expression library

mRNA was isolated from *T. solium* metacestodes using the Fast-Track System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A sample containing 5 μg of mRNA was converted into double stranded cDNA with the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) and then ligated to the λ-ZAP expression vector using Uni-ZAP® XR library kit (Stratagene). The original library was amplified and contained 2×10^{10} pfu/ml and 4% of non-recombinant phages.

2.6. Genomic library

Purified genomic DNA (gDNA) from *T. solium* metacestodes was supplied by Dr. Sciutto from the Department of Immunology, Instituto de Investigaciones Biomédicas, Universidad Autónoma de Mexico, Mexico. Five μg of gDNA were partially digested with *Xho*I (five units, 1 h, 37 °C) (Promega, Madison, WI, USA) to yield products with a molecular mass from 9 to 23 kb. A 2-μg aliquot of the digested gDNA was ligated into λ-FIX® II vector (Stratagene) and then packaged using Gigapack III Gold Packaging system (Stratagene). The original library was amplified and contained 1.2×10^{11} pfu/ml and 9% non-recombinant phages.

2.7. Screening of the libraries

The cDNA expression library was screened using sera from rabbits immunized with different extracts of *T. solium* and *T. saginata* metacestodes, versus normal rabbit serum [16]. Bound total rabbit Igs were revealed with goat anti-rabbit Ig-alkaline phosphatase (H and L chains) (Pierce, Rockford, IL, USA) and visualized using NBT (75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide) (Sigma–Aldrich, St. Louis, MO, USA) and BCIP (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide) (Sigma–Aldrich). The positive clones were purified by the same serum selection procedure and finally subjected to in-vivo excision by helper phage rescue [15] to generate pBluescript plasmids.

The *T. solium* genomic library was screened according to Sambrook et al. [16], using the F18 cDNA, isolated during the screening of the mature metacestode expression library. Four replica nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) plaque lifts were prepared from recombinant phage-infected *E. coli* XL1-Blue MRA strain, and the phage DNA was allowed to hybridise with digoxigenin-11-dUTP-labelled F18 cDNA (Roche Diagnostics, Mannheim, Germany). Hybridisations were carried out under high stringency conditions at 68 °C, overnight. After hybridisation, the filters were washed at room temperature for 10 min in 2× SSC buffer (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7.0) (Sigma–Aldrich) and 0.1% (w/v) sodium dodecylsulphate (SDS) (Sigma–Aldrich). Immunodetection was carried out with antidigoxigenin conjugate coupled to alkaline phosphatase, and the immune complexes were visualized with NBT (75 mg/ml) and BCIP (50 mg/ml) (Sigma–Aldrich), by colorimetric detection. Positive clones were recovered and further studied by restriction digestion and Southern hybridisation (see below). The selected genomic fragments were subcloned into pBluescript KS^+, according to standard methods [15] and then sequenced.

2.8. Digestion and electrophoresis of T. solium gDNA

The genomic organization of the F18 gene was examined as follows: 8-μg aliquots of *T. solium* gDNA were digested to completion with *Alu*I, *Eco*RI, *Pst*I, *Rsa*I, *Bam*HI, *Hae*III and *Hin*dIII (Roche Diagnostics) using the procedures recommended by the manufacturers. Electrophoresis of the digested DNA samples and the DIG II and molecular mass markers (Roche Diagnostics) were simultaneously electrophoresed in 0.7% agarose gel.

2.9. Southern blotting, labelling, and hybridisation procedures

The electrophoresis of the digested DNA samples was transferred to positively charged nylon membranes (Roche Diagnostics), which were carried out

by standard procedures [17]. The F18 cDNA probes were nonradioactively labelled with digoxigenin-11-dUTP (Roche Diagnostics) by a random oligonucleotide primer method, according to the manufacturer's instructions. Hybridisations were conducted overnight under high-stringency conditions at 68 °C. After hybridisation, the filters were washed at 68 °C for 10 min, in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and then for a further 40 min in 0.1× SSC–0.1% SDS. The immunodetection was carried out with antidigoxigenin conjugated with alkaline phosphatase, and the immune complexes were visualized using the chemiluminescence substrate CSPD (Roche Diagnostics) on X-ray film (Amersham Biosciences, Uppsala, Sweden), with an intensifying screen at room temperature for 15 min, as described in the manufacturer's instructions.

2.10. DNA sequencing

The DNAs from the recombinant plasmids to be sequenced were prepared using the Qiagen Plasmid Mini Kit (Qiagen, Valencia, CA, USA) and the dideoxy chain termination sequencing reaction [18] was performed using Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Langen, Germany). Sequencing reactions were automatically analysed on an Applied Biosystems 3700 DNA sequencer (Perkin-Elmer). The bioinformatic program GENSCAN [19] was used to predict a possible F18 open reading frame and the organization of the F18 gene. PSIPRED [20], PROSITE [21] and DAS [22] bioinformatic programs were used to predict the secondary structure of F18 protein. DNA sequences and predicted amino acid sequence comparisons were carried out with the EMBL and SWISS-PROT databanks, respectively, using software packages from the Genetic Computer Group [23].

2.11. DNA amplification

The extension of the 5′ end of the F18 cDNA was obtained by PCR, using standard PCR protocols and maxipools prepared from aliquots of the amplified *T. solium* metacestodes expression library [24]. The primers used were: T3 (5′ AATTAACCCTCACTAAAGGG 3′), forward primer from λ-Zap vector, and the reverse primer PSf18R2 (5′ CGAGACAGAATCTCCTCGAGCTGCGCC 3′) which derives from the known F18 cDNA sequence. The amplified products were subcloned into pGEM-T vector (Promega) and then sequenced as described above.

The full F18 genomic sequence was obtained by PCR, using the Expand™ Long Template PCR system (Roche Diagnostics) and *T. solium* gDNA. The primers prepared from F18 cDNA were: the forward primer, PF18genF1 (5′ GGTCGTATTACCGATCTCAAAGCA 3′) and the reverse primer, PF18genR1 (5′ GAAGGTAGTTAGGTGGGTGTGGGG 3′). The primers prepared from a known F18 genomic sequence were: the forward primer, PF18genF3 (5′ GGGAGTGGTGTCTTCTGTCAATG 3′) and the reverse primer, PF18genR4 (5′ GAGCGTTTTCAAGCCCGACAGAC 3′). The reaction was carried out following the manufacturer's instructions. Working conditions for the PCR were 94 °C for 1 min (initial denaturation), followed by 30 cycles at 94 °C/30 s, 65 °C/30 s and 68 °C/2.5 min and 68 °C/7 min (final extension). The amplified products were subcloned into pGEM-T vector (Promega) and sequenced.

All the amplification products were separated on 0.7% agarose gels and were visualized under UV light by ethidium bromide staining. Oligonucleotide primers were synthesized by Roche Diagnostics. PCR experiments were performed on a programmable Perkin-Elmer GeneAmp TM PCR System 2400.

2.12. Expression and purification of recombinant full-length and carboxyl terminal fragments of F18 cDNA

The full length F18 cDNA in pBluescript vector was used as a template to amplify F18 fragment by PCR, using specific oligonucleotides.

The sense F18PROF4 (5′ GAGAGAGAGAATTCCTGAACGGTCGT 3′) and antisense F18PROR2 (5′ GAGAGAGAGTCGACGGTTATATAACT 3′) primers included *Eco*RI and *Sal*I sites (underlined). The F18 PCR product was gel purified and cloned into the expression plasmid vectors pGEX-4T-1 (Amersham Biosciences) downstream of glutathione-S-transferase (GST) and pQE-31 (Qiagen) which can be produced by placing the 6×-His tag at the N-terminus of the protein of interest. Three different

and overlapping carboxyl-terminal fragments derived from F18 cDNA were amplified by PCR with three different forward primers with an *Eco*RI site: F18-51aaF1 (5′ GAGAGAGAGAATTCGACACAGCG-CCA 3′), F18-80aaF1 (5′ GAGAGAGAGAATTCC-ACAAAGGTGAG 3′), F18-97aaF1 (5′ GAGAGA-GAGAATTCCATAGCTGTTCCAAG 3′) and the same reverse primers with a *Sal*I site: F18aaR1 (5′ GAGAGAGAGTCGACAGCTGGGGTCTC 3′). The PCR products were subcloned into pGEX-4T-1 (Amersham Biosciences) and purified as described previously. Conditions for the PCRs were 94 °C for 1 min (initial denaturation), followed by 30 cycles at 94 °C/30 s, 65 °C/30 s and 72 °C/2 min and 72 °C/7 min (final extension).

E. coli strain BL21 were transformed with pGEX-4T-1-F18, pGEX-4T-1-F18-51aa, pGEX-4T-1-F18-80aa or pGEX-4T-1-F18-97aa constructions. The induction of the F18 molecules in pGEX vector was carried out with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich) and 100 μg/ml ampicillin (Sigma–Aldrich) in LB medium at 37 °C, during 3 h. For the purification of the recombinant proteins (G-F18, G-F18-51aa, G-F18-80aa and G-F18-97aa), a Bulk GST purification Module (Amersham Biosciences) and a B-PER® Bacterial Protein Extractor Reagent (Pierce) were used in order to improve the efficiency and quality of the purification as described by the manufacturers. *E. coli* strain M15 [pREP4] was transformed with pQE-31-F18 construction. The induction of the F18 molecule in pQE-31 vector was carried out in LB medium at 37 °C, during 4 h, with 1 m*M* IPTG, 200 μg/ml ampicillin and 25 μg/ml kanamycin (Sigma–Aldrich). The protein His-F18 was purified with the NI-NTA Spin Kit (Qiagen) under denaturing conditions as described by the manufacturers. The purified proteins were checked by 10% and 12.5% SDS–PAGE and Coomassie blue staining. Protein concentrations were determined with a BCA protein assay kit (Pierce).

2.13. Synthesis of peptides derived from F18 molecule-deduced amino acid sequence

Three peptides derived from the F18-deduced amino acid sequence were designed according to the Jameson–Wolf antigenicity index [25]. The peptides were synthesized in the Department of Organic Chemistry of Barcelona University (Barcelona, Spain) and their sequences were: F18pep-1 **C**-$_{10}$LKSELENKKDGENAE$_{23}$, F18pep-2 **C**-$_{67}$KTEATSLQQRNTE$_{79}$ and F18pep-3 $_{144}$HKGEQIPDEATC$_{155}$. A cysteine residue, which is marked in bold, was added to each peptide to facilitate their coupling to carrier proteins.

2.14. ELISA with recombinant proteins and peptides

Microplate wells of Maxisorb® 2 flat bottom Nunc (Nalgene Nunc, Rochester, NY, USA) were coated with 1 μg of G-F18, His-F18, G-F18-51aa, G-F18-80aa, and G-F18-97aa purified proteins. *T. solium* cyst fluid (1 μg) (as a positive control) and GST (1 μg) were used in the same conditions. The microplate wells were coated with 0.2 μg of the peptides F18pep-1, F18pep-2 and F18pep-3. After overnight incubation at 4 °C, the wells were blocked with 1% bovine serum albumin (BSA) (Sigma–Aldrich) diluted in phosphate-buffered saline (PBS), for 1 h at 37 °C. After washing with PBS containing 0.05% Tween® 20 (Sigma–Aldrich) (PBS-T), three times during 5 min, 100 μl of serum samples diluted at 1/100 in PBS were added and incubated for 1 h at 37 °C. Each sample was run in duplicate. After washing with PBS-T, three times during 10 min, 100 μl of goat anti-human IgG conjugated with peroxidase (H and L chains) (Pierce) diluted 1/5000 were added and incubated for 1 h at 37 °C. Plates were washed four times during 10 min with PBS-T, and 100 μl of substrate 2,2′-azino-bis 3-ethylbenthiazoline-6 sulfonic acid (ABTS) (Sigma–Aldrich) in 10 ml citrate–phosphate–perborate buffer (Sigma–Aldrich) were added and the plates were incubated for 30 min at room temperature. The absorbances were determined in an ELISA reader (Dynatech MR 700, Lorton, VA, USA) at 405 nm. The cut-off was established using the media of 35 non-reactive serum samples plus three standard deviations (SD).

2.15. Preparation of rabbit F18-specific antiserum

Two female New Zealand White rabbits were each injected on days 0, 21 and 49 with 200 μg of

purified recombinant H-F18 protein. On the 0 day, before the first immunization, 20 ml were obtained from each rabbit as a negative control. The first injection was subcutaneous and contained 500 μl of complete Freund's adjuvant (Becton Dickinson, Franklin Lakes, NJ, USA), the following injections were intramuscular and contained 500 μl incomplete Freund's adjuvant (Becton Dickinson). Five days after the third immunization, the animals were bled. The serum obtained will be referred to as anti-F18. The serum title was determined by ELISA.

2.16. SDS–PAGE and Western blot analysis

One-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting of purified GST-F18 was carried out using 10% polyacrylamide gels. The separated protein was transferred to nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotting apparatus (Fasblot Biometra, Göttingen, Germany) during 1 h at 80 mA. Later, the filters were blocked with PBS containing 3% BSA at 4 °C, overnight. Then, the membranes were incubated with anti-F18 rabbit sera diluted 1/10,000 in the blocking solution, for 1 h at 37 °C. After washing, goat anti-rabbit IgG conjugated with alkaline phosphatase (H and L chains, Pierce) diluted 1/5000 was added and incubated for 1 h at room temperature. The filters were developed using NBT (75 mg/ml) and BCIP (50 mg/ml in dimethylformamide).

2.17. Nucleotide sequence accession numbers

The GenBank accession numbers of the F18 cDNA and F18 gene sequences are AJ493440 and AJ510265, respectively.

3. Results

3.1. Isolation and analysis of T. solium F18 cDNA

To identify immunodiagnostic antigen genes, a *T. solium* metacestode expression cDNA library was prepared and immunoscreened with sera from rabbits immunized with a mix of taeniid antigens.

The antibody screening of 1.3×10^6 clones from the *T. solium* metacestodes cDNA library yielded 40 promising signals. Of these, one molecule of 0.9 kb (F18) was sequenced and further characterised. Using the Genescan program, the F18 cDNA has an 83-nucleotide 5′-untranslated region, a 690-nucleotide open reading frame, and a 184-nucleotide 3′-untranslated sequence. The open reading frame encodes a putative polypeptide of 229 amino acids with a 26.06×10^3 M_r theoretical molecular mass, and an isoelectric point of 6.35 (Fig. 1A). Using the bioinformatic program ProSite a putative N-glycosylation site of F18 protein was identified in the residue 138 (NLTV) (Fig. 1A). Study of the predicted secondary structure using the PSIPRED program revealed that F18 was a hydrophilic protein with mainly α-helices and with β-sheets, turns and random coils. No transmembrane domain was found by the DAS bioinformatic program.

The DNA sequences and predicted amino acid sequence comparisons were carried out with the EMBL and SWISS-PROT databanks, using software packages from the Genetic Computer Group. No significant similarities were found between the F18 sequence and other sequences included in the databases.

3.2. Amplification of 5′ end of the F18 cDNA

We tried to amplify the 5′ end of the F18 cDNA with a PCR-cloning approach, using standard PCR protocols and maxipools prepared from aliquots of the amplified *T. solium* metacestodes expression library, as well as primers derived from both the λ-Zap vector and the known F18 sequence (T3 and PSf18R2). The ORF of the F18 cDNA was confirmed by the characterisation of the 5′ UTR (Fig. 2A). In most of the cases, fragments with a size bigger than the F18 sequence already characterized were amplified. These PCR products from the cDNA library were only extended by 14 more nucleotides and possessed an inserted G as the first nucleotide, being the putative capping G found on eukaryotic mRNA [26,27].

3.3. Obtaining the full sequence of the F18 gene

Two strategies were used to obtain the complete F18 sequence: (i) the amplified *T. solium* genomic

A

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MSSEYSTMDLKSELENKDGENAELKAQLEEILSRFKDLKIKAERRAKLLH  50
HVMSEFNAMTKEYDILKTEATSLQQRNTEMYDHIVNQDTRIDQLEQSIQV 100
LEAENNELRRSRIIDASTKQWYGECTHSCSKHPRTYPNLTVHGHKGEQIP 150
DEATCPPCETCSEAEAWAQGLMDTAPKFTDSLRKHFQSTLPLRSRGTLPR 200
SMQSTPASTLKKPSSVPAQETPAPAYKVN*                      229
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B

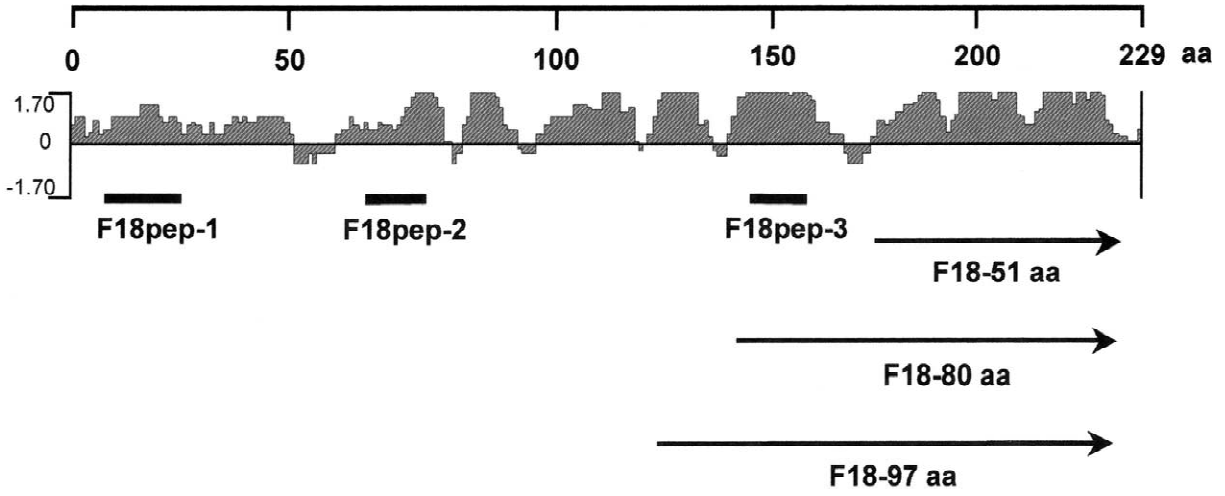

Fig. 1. (A) Full length amino acid sequence of F18. Amino acid numbers are written on the right of the sequence. The putative glycosylation site is shown in a box. Asterisk represents the termination codon. (B) Antigenic index of the F18 deduced amino acid sequence. The 229 amino acid sequence deduced from the F18 sequence are represented by the upper ruler and the protein antigenic index, calculated according to Jameson and Wolf [23], by peaks. Scheme showing the location of the F18pep-1, F18pep-2 and F18pep-3 peptides and G-F18-55aa, G-F18-80aa and G-F18-97aa fusion proteins within the F18 amino acid sequence. The peptides and fusion proteins are indicated by bars and arrows, respectively.

library was screened using a digoxigenin-labelled full length F18 cDNA as a probe, under conditions of high stringency. Two positive signals were obtained after screening 100,000 recombinant clones and the positive clones purified and further characterized. The two purified clones showed the same *Eco*RI restriction patterns and both contained two bands of 4.0 kb and 1.1 kb genomic fragments, which strongly hybridised with the F18 cDNA probe (Fig. 3B). The sequencing of the two fragments, the 4.0 kb and 1.1 kb subcloned into pBluescript KS^+, revealed that these fragments did not contain the complete F18 genomic sequence (Fig. 2A). The 4.0 kb fragment contained a portion of the first intron, the full first exon and the putative regulatory elements in the 5′-flanking region of the F18 gene. The 1.1 kb fragment contained a part of the first intron, the full second exon and a portion of the second

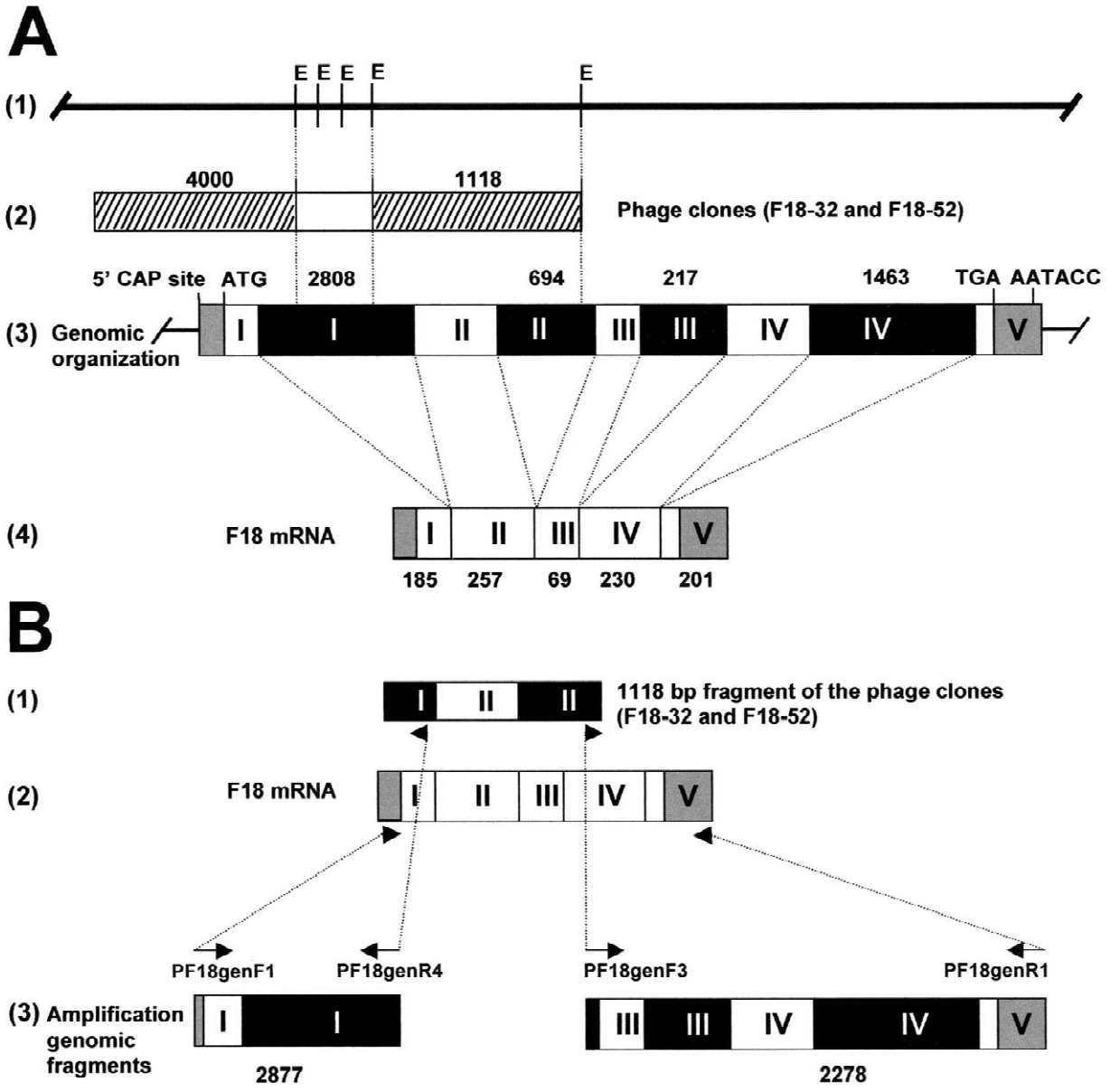

Fig. 2. (A) Organization of the *T. solium* F18 gene. (1) Line represents the genomic locus with *Eco*RI (E) restriction sites. (2) Line shows the two lambda clones that contain a gene portion and their names. The cross-hatched areas indicated regions subcloned and sequenced. The size of two fragments (bp) is indicated. (3) Line represents the number and position of exons (open box) and introns (black box) in the gene. The distribution of exons and introns was determined by aligning the cDNA with the genomic sequences. 5′ CAP site, iMet-ATG, termination codon-TGA, polyadenylation signal-AATACC positions are marked. The sizes of introns (bp) are indicated. (4) Line represents the mRNA. The size of exons (bp) is indicated. (B) Illustration showing the strategy of genomic PCR. (1) Line represents the 1.1 kb genomic fragment of the clones lambda which strongly hybridised with the F18 cDNA probe. (2) Line represents the F18 mRNA. (3) Line represents the sizes (bp) and F18 amplification genomic products. In all the lines, location of the PF18genF1, PF18genF3, PF18genR1 and PF18genR4 oligonucleotide within the 1.1 kb genomic fragment of the phage clones and F18 mRNA. The primers are indicated by arrows in the scheme.

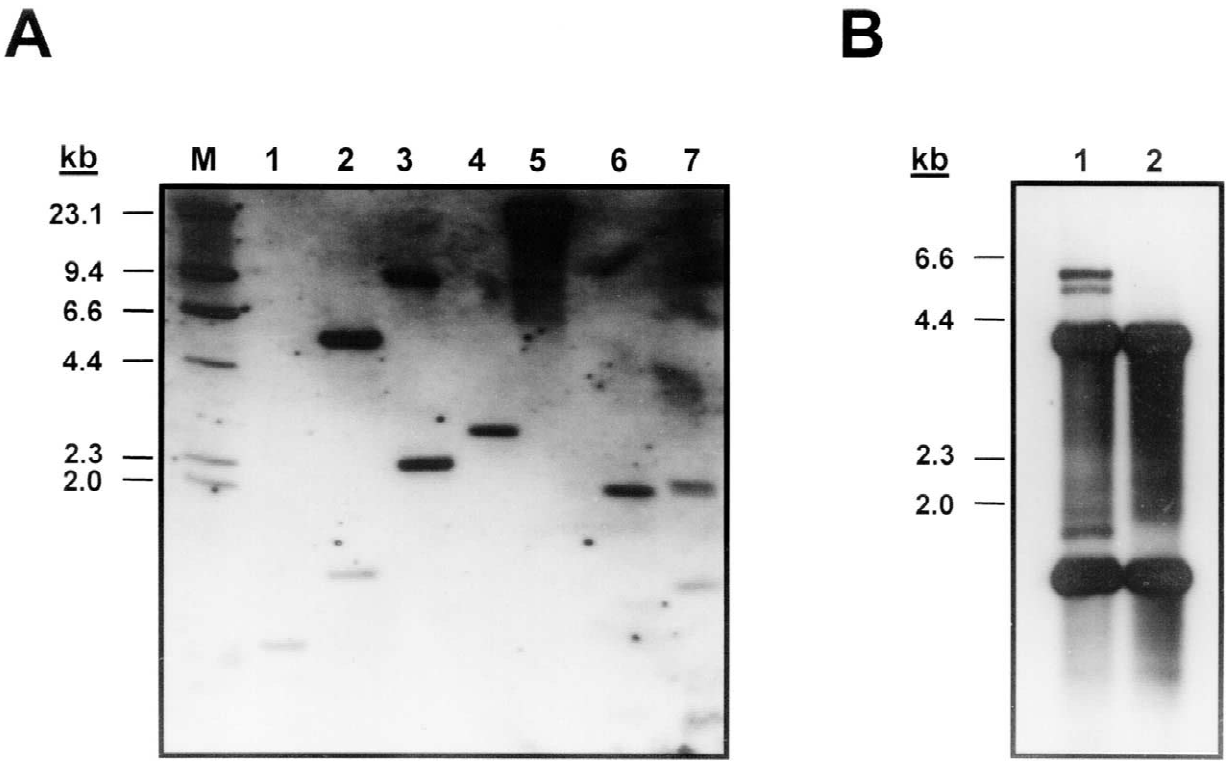

Fig. 3. (A) Restriction and hybridisation analysis of *Taenia solium* total DNA. The *T. solium* genomic DNA (8 μg per lane) digested with *Alu*I (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3), *Rsa*I (lane 4), *Bam*HI (lane 5), *Hae*III (lane 6) and *Hin*dIII (lane 7), were electrophoresed on 0.7% agarose gel, blotted and hybridised to the digoxigenin-11-UTP-labelled F18 cDNA insert. (B) Restriction and hybridisation analysis of λF18-32 and λF18-52 DNA. Five μg of λF18-32 DNA (1) and λF18-52 DNA (2) were digested with *Eco*RI and analysed as in (A). Size of DNA fragments was determined using DNA molecular-size standards (DIG II molecular mass markers, Roche Diagnostics) (lane M). The numbers on the left indicate the sizes (in kb) of the molecular mass markers.

intron. (ii) In order to obtain the complete F18 sequence, we tried with a PCR-cloning approach, using Long Expand PCR protocols (Roche Diagnostic) and *T. solium* gDNA, and primers derived from both the 5′ and 3′ ends of F18 cDNA (PF18genF1 and PF18genR1) and 5′ and 3′ ends of the 1.1 kb genomic fragment (PF18genF3 and PF18genR4) (Fig. 2B). Two PCRs were carried out with different sets of primers. In the first PCR, using PF18genF1–PF18genR4, as primers, a band of 2.8 kb was obtained. In the second PCR we used PF18genF3–PF18genR1 as primers, and a band of 2.2 kb was obtained. The sequencing of both fragments revealed the full F18 genomic sequence (Fig. 2B).

3.4. Organization of the F18 gene

The comparison of the gDNA and cDNA sequences of the F18 molecule revealed that the full *T. solium* F18 genomic sequence occupied a stretch of 6.1 kb, with four introns (2808 bp, 694 bp, 217 bp and 1463 bp) separated by five exons (185 bp, 257 bp, 69 bp, 230 bp and 201 bp). These results were confirmed by the Genescan bioinformatic program. The locations of exons and introns, CAP sites, translation start codon (iMet), poly (a)-tail signals and stop codon, are shown in Fig. 2A [28–30]. When Southern analysis was carried out with *T. solium* gDNA digested by *Alu*I, *Eco*RI, *Pst*I, *Rsa*I, *Bam*HI,

*Hae*III and *Hin*dIII, using the F18 cDNA as a probe, the hybridisation patterns obtained suggested that the F18 gene is a single copy gene (Fig. 3A). Furthermore, the sizes of the bands are consistent with the sequence of the F18 gene determined in the present study.

3.5. Expression and purification of recombinant H-F18 and G-F18 proteins

The F18 cDNA was subcloned into pQE-31 and pGEX-4T-1 expression vectors. The plasmid construct (pQE-31-F18) was transformed into *E. coli* strain M15 [pREP4]. The fusion protein had around 31×10^3 M_r, because of the histidine tag (Fig. 4A). The H-F18 recombinant protein was purified with a Ni-NTA affinity column, that contains a metal chelate chromatography material (NI-NTA silica), which is used to purify recombinant proteins carrying a small affinity 6×His tag. The F18 recombinant protein was purified under denaturing conditions and then was used in rabbit immunization assays (Fig. 4A). The anti-F18 serum identified the H-F18 protein in ELISA and Western-blot assays (data not shown). *E. coli* strain BL21 was transformed with pGEX-4T1-F18 and yielded a GST-F18 fusion protein with a molecular mass of about 57×10^3 M_r, which was the expected size (Fig. 4B). The G-F18 recombinant protein was purified under native conditions. Bacterial cultures were lysed and loaded onto a glutathione–Sepharose 4B column. The glutathione coupled to Sepharose 4B was used for purification of fusion proteins containing the GST protein. Then, the bound material eluted from the matrix with glutathione buffer. The purified product was observed on a Coomassie blue-stained SDS–PAGE gel, and in addition to the 57×10^3 M_r fusion protein, more truncated forms of F18 protein appeared (Fig. 4B), which were identified by the anti-F18 rabbit sera (Fig. 4B).

3.6. Expression and purification of recombinant G-F18-51aa, G-F18-80aa and G-F18-97aa proteins

The F18 cDNA was used as a template to amplify the carboxyl-terminal fragment from the F18 molecule using the forward primer F18-51aaF1 and the reverse primer F18aaR1. The deduced amino acid sequence from the 153-bp PCR product was equivalent to the last 51 amino acids from the carboxy-terminal F18 protein. PCR reaction with primers F18-80aaF1 and F18aaR1 amplified a 240-bp fragment. The deduced amino acid sequence from the 240-bp PCR product was equivalent to the last 80 amino acids from the carboxy-terminal F18 protein. PCR reaction with primers F18-97aaF1 and F18aaR1 amplified a 291-bp fragment. The deduced amino acid sequence from the 291-bp PCR product was equivalent to the last 97 amino acids from the carboxy-terminal F18 protein. The PCR products were subcloned into pGEX-4T1, expressed and purified as described previously for G-F18. The fusion proteins were called G-F18-51aa, G-F18-80aa and G-F18-97aa. Expressions of G-F18-51aa, G-F18-80aa and G-F18-97aa were demonstrated by three bands with molecular masses around 31×10^3 M_r, 34×10^3 M_r and 36×10^3 M_r, respectively, on a Coomassie blue-stained SDS–PAGE gel (Fig. 4C).

3.7. ELISA analysis

ELISA analyses were developed to evaluate the diagnosis properties of the F18 recombinant proteins and derived peptides. ELISAs were carried out using sera from 50 NCC seropositive patients (30 patients from endemic areas of Venezuela and 20 Latin America immigrants, who now live in Spain). When the sera were examined by ELISA using as antigens G-F18, H-F18, G-F18-51aa, G-F18-80aa, G-F18-97aa, F18pep-1, F18pep-2 and F18pep-3, the sensitivities of the different molecules were 86%, 58% 18%, 44%, 24%, 38%, 6% and 14%, respectively (Table 1). Moreover, when ELISAs were carried out with 22 sera from patients with acute NCC, the recombinant proteins and peptides showed a low sensitivity. The sensitivity of G-F18, H-F18, G-F18-80aa and G-F18-97aa recombinant proteins was 4.5% (1/22) and 0% for G-F18-51aa recombinant protein, F18pep-1, F18pep-2 and F18pep-3 peptides (Table 1).

When the molecules were analysed with sera from patients with hydatidosis (14), fasciolasis (9) and schistosomiasis (10) the specificity of the ELISAs was 100% (Table 1) with respect to both hydatidosis and fasciolasis patients, but it was lower when schistosomiasis patients were checked. When all

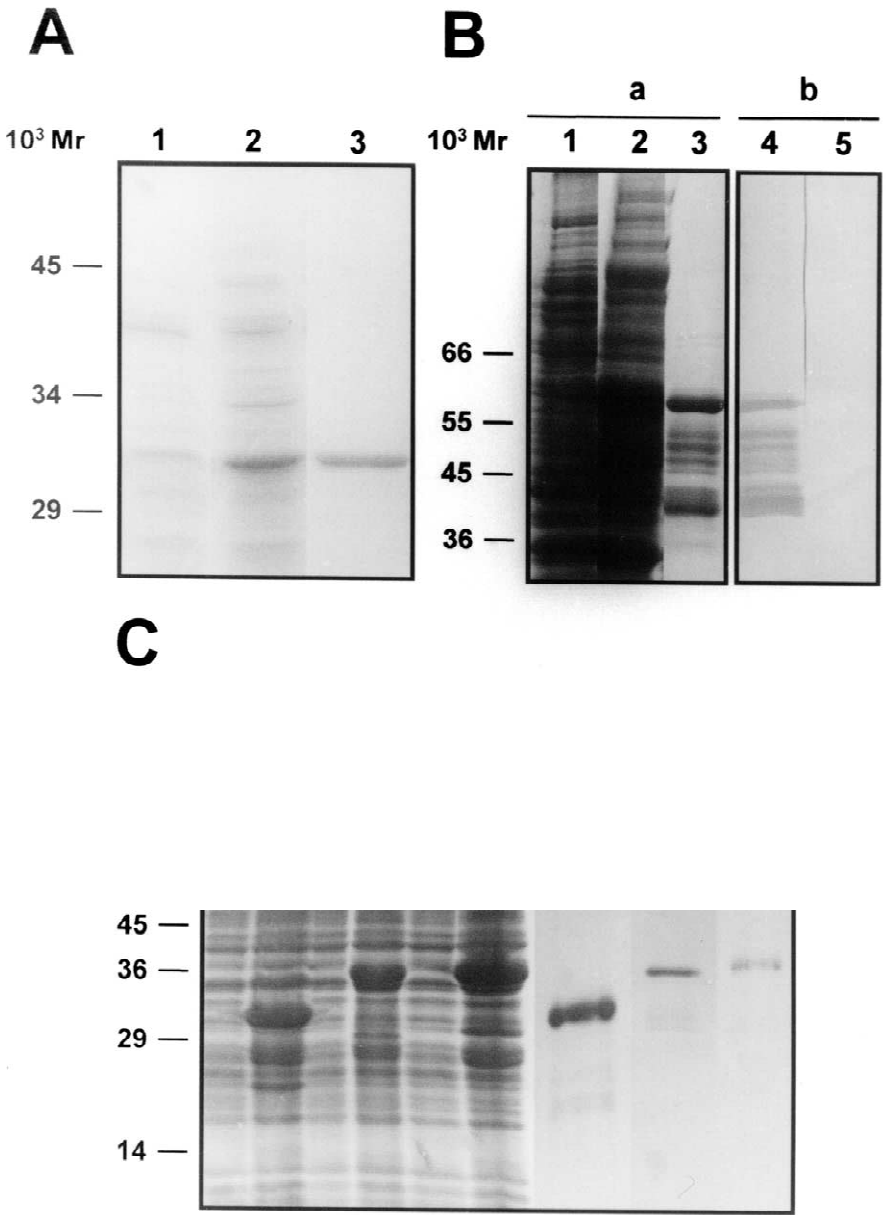

Fig. 4. (A) SDS–PAGE analysis of recombinant H-F18 preparations. Lane 1, non-induced pQE31-F18-transformed crude cell extract; lane 2, pQE31-F18-transformed crude cell extract and induced with 1 m*M* IPTG; lane 3, purified H-F18 fusion protein. (B) SDS–PAGE analysis of recombinant GST-F18 preparations (a). Lane 1, non-induced pGEX-4T-1-F18-transformed crude cell extract; lane 2, pGEX-4T-1-F18-transformed crude cell extract and induced with 1 m*M* IPTG; lane 3, purified G-F18 fusion protein. Western-blot analysis of recombinant G-F18 (b). Purified G-F18 fusion protein was analyzed by SDS–PAGE and probed with specific anti-F18 sera (lane 4) or rabbit normal (lane 5). (C) SDS–PAGE analysis of truncated recombinant G-F18 preparations: (c) G-F18-51aa, (d) G-F18-80aa and (e) G-F18-97aa. Lane 1, non-induced crude cell extract; lane 2, induced crude cell extract with 1 m*M* IPTG; lane 3, purification of the truncated G-F18 fusion proteins. (M_r) Molecular mass markers, the numbers indicate the sizes (in 10^3 M_r) of the molecular mass markers.

Table 1
Results of sensitivity and specificity obtained by ELISA with cyst fluid from *T. solium* (CF-Ts); G-F18 and H-F18 recombinant proteins; G-F18-55aa, G-F18-80aa and G-F18-97aa truncated recombinant proteins; F18pep-1, F18pep-2 and F18pep-3 peptides using serum samples from 50 patients with NCC, 20 patients with acute NCC (a-NCC) and 33 serum samples from patients with other parasite infections: hydatidosis (14), fasciolasis (9) and schistosomiasis (10)

%	Proteins product	CF-Ts	G-F18	H-F18	G-F18 51aa	G-F18 80aa	GF18 971aa	F18 pep-1	F18 pep-2	F18 pep-3
Sensitivity	NNC	100%	86%	58%	18%	44%	24%	38%	6%	14%
	(n=50)	50/50	43/50	(29/50)	(9/50)	(22/50)	(12/50)	(19/50)	(3/50)	(7/50)
	a-NNC	100%	4.5%	4.5%	0%	4.5%	4.5%	0%	0%	0%
	(n=22)	22/22	1/22	1/22	0/22	1/22	1/22	0/22	0/22	0/22
Specificity	Hydatidosis	7%	100%	100%	100%	100%	100%	100%	100%	100%
	(n=14)	13/14	0/14	0/14	0/14	0/14	0/14	0/14	0/14	0/14
	Fasciolasis	100%	100%	100%	100%	100%	100%	100%	100%	100%
	(n=9)	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
	Schistosomiasis	80%	90%	80%	70%	50%	50%	40%	60%	50%
	(n=10)	2/10	1/10	2/10	3/10	5/10	5/10	6/10	4/10	5/10

cysticercosis patients' sera were analysed by *T. solium* cyst fluid antigen, the assays showed a sensitivity of 100%. However, 13 sera from patients with hydatidosis and two with schistosomiasis recognised cyst fluid antigens (7% and 80% of specificity, respectively). On the other hand, none of the human sera recognized GST, which suggests that the presence of GST domain in the recombinant protein did not affect the antigenicity detected in the assays designed [11].

4. Discussion

Human NCC is a serious parasitic disease of the central nervous system. The principal difficulty for evaluating results and planning therapeutic strategies continues to be the clinical polymorphism of NCC; in almost every patient the disease has its own particular course depending on personal immune response to NCC, the severity of infestation, the location of cysts, and the site and number of neurological lesions [31]. The development of an immunological test that detects specific antibodies either in sera or in cerebrospinal fluid (CSF) would provide a reliable and specific adjuvant for the diagnosis of NCC [32]. Regarding this, some antigenic components of *T. solium* metacestodes, which have been analysed in depth, have great relevance in the serological diagnosis of NCC, although immunological detection of some forms of NCC continues to be an unresolved problem [8,11,33–39]. So, the purpose of this work was the searching and characterization of new *T. solium* metacestode molecules with diagnostic properties and their use in the detection of NCC by an immunodiagnostic method. The construction and immunoscreening of a *T. solium* metacestodes expression library was carried out and a cDNA, called F18, was selected and further characterized. F18 cDNA had an open reading frame with 690 nucleotides, which encoded a putative polypeptide of 229 amino acids with an isoelectric point of 6.35. Moreover, a putative N-glycosylation site of F18 protein was identified in the residue 138. The predicted molecular mass of the F18 protein based only on the full length F18 mRNA was 26.06×10^3 M_r. It will be interesting to know the real molecular mass of F18 through the identification of the native molecule in *T. solium* metacestodes. Probably, the parasite native F18 protein may exhibit a bigger size due to the putative N-glycosylation site [10,11] and other potential post-translational modifications.

In order to undertake the genomic characterization of the F18 gene, two strategies were employed: (i) an F18 cDNA hybridisation screening of a *T. solium* genomic library and (ii) a PCR-cloning using primers derived from the known F18 sequence. By these strategies, it was determined that the *T. solium* F18 gene spans ~6.1 kb and contains four introns separated by five exons. The gene structure showed the standard features of a eukaryotic protein-coding

gene. However, the F18 gene contains a putative distinct polyadenylation signal sequence. This is not unique to F18 since other variants have been observed in some parasite genes already characterised [27,40]. In the future, primer extension analysis of the 5′ end of the *T. solium* F18 gene would be desirable in order to find the transcription initiation site of the gene. Also, to estimate the copy number of the F18 gene per genome, Southern blot analysis was performed and the results supported the fact that F18 is a single copy gene, with DNA fragments of the predicted molecular size after digestion with specific restriction enzymes.

Regarding expression of the F18 cDNA, and previous to the immunological studies with the molecule, two different expression vectors, pGEX-4T-1 and pQE-31, were used. The recombinant proteins obtained were called G-F18 (57×10^3 M_r) and H-F18 (31×10^3 M_r) respectively, and the last was used in the rabbit immunization to get an anti-F18 specific sera. When the recombinant protein G-F18 was observed in a stained 10% SDS–PAGE, in addition to the 57×10^3 M_r fusion protein, more truncated forms of F18 protein appeared, which were identified by the anti-F18 rabbit sera. The presence of these truncated forms is difficult to understand. Eukaryotic proteins expressed in *E. coli* may be susceptible to bacterial proteases [41], however, we used *E. coli* strain BL21, which is deficient in proteases, during the G-F18 expression in order to avoid these activities. On the other hand, expression of eukaryotic genes in prokaryote organisms can be affected by codon usage due to differences in abundance of tRNA species [42,43]. In relation to the expression of the H-F18 using pQE vector, full protein as well as truncated forms were also detected, although the total yield expression was less than with the pGEX system.

In addition to the full F18 recombinant proteins, and considering the high antigenicity of the molecule carboxy end [25], truncated fusion proteins corresponding to this region were prepared. The recombinant proteins G-F18-51aa (31×10^3 M_r), G-F18-80aa (34×10^3 M_r) and G-F18-97aa (36×10^3 M_r) were expressed using pGEX-4T-1 as a vector and purified. Also, three peptides, called F18pep-1, F18pep-2 and F18pep-3, were designed (Fig. 1B).

In order to test the diagnostic properties of the purified protein products (G-F18, H-F18, G-F18-51aa, G-F18-80aa, G-F18-97aa) and designed peptides (F18pep-1, F18pep-2 and F18pep-3), ELISAs were carried out with 22 sera from patients with acute NCC and 50 sera from patients with unclassified NCC. All samples were previously tested by ELISA, using cyst fluid from *T. solium* metacestodes as antigen. In addition, sera from other helminth diseases were used.

The ELISA results with F18 molecules showed a very low sensitivity with sera samples from patients with acute NCC, in contrast to the results from the sera of the other NCC patients. Thus, the G-F18 fusion protein yielded the best results, with 86% of sensitivity (43/50) and 100% of specificity with sera from hydatidosis and fasciolasis patients, and a lower specificity with sera from schistosomiasis patients (Fig. 5).

With respect to ELISAs carried out with G-F18-51aa, G-F18-80aa and G-F18-97aa overlapping recombinant products, and F18pep-1, F18pep-2 and F18pep-3 peptides, using sera samples from NCC patients, the sensitivities of these antigens were lower than the one obtained with the full G-F18 protein. On the other hand, ELISAs show that the sensitivity of H-F18 (58%) was a bit lower than the sensitivity of G-F18 protein (86%). The sensitivity differences observed could be explained considering

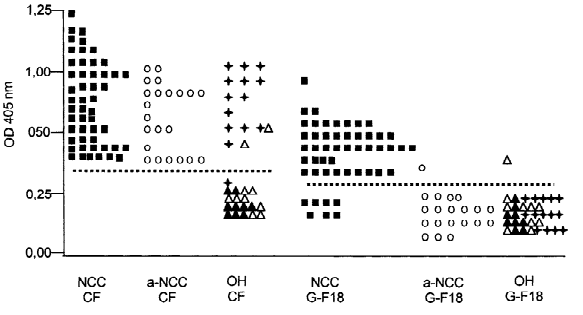

Fig. 5. Results of ELISA assays with samples from 50 patients with NCC (■) using cyst fluid from *T. solium* (NCC-CF) and G-F18 recombinant protein (NCC-G-F18). Twenty-two sera samples from patients with active NCC (○) using cyst fluid from *T. solium* (aNCC-CF) and G-F18 recombinant protein (aNCC-G-F18). Thirty-three sera samples from patients with other helminth infections: (+) hydatidosis (14), (△) schistosomiasis (10) and (▲) fasciolasis (9), using cyst fluid from *T. solium* (OH-CF) and G-F18 recombinant protein (OH-G-F18). (. . .) Cut-off of CF= 0.361 and G-F18=0.328; OD, optical density.

that H-F18 protein was purified under denaturing conditions, and the Protein Refolding Recommendations of the manufacturer, Qiagen, suggests that it is generally preferable to be able to purify recombinant proteins under native conditions, but for many proteins, particularly when large yields are required, this may not be always possible. We think that renaturing procedures for H-F18 recombinant protein should be necessary to obtain a suitable refold of the protein, before its use in ELISA.

Also, and in relation with the antigenicity/sensitivity of the F18 molecule, several studies about glycoproteins from *T. solium* metacestodes reported that these parasite proteins were specific to NCC [8,39,44], and F18 protein shows a putative N-glycosylation site, so F18 could be a glycoprotein of *T. solium* metacestodes. In relation with this, Obregón-Henao et al. [45] suggested that the antigenicity of glycoproteins of *T. solium*, such as 18×10^3 M_r TSG [36], was due to a combination of carbohydrate and protein epitopes. So, native 18×10^3 M_r TSG was more sensitive for detecting anti-cysticercosis antibodies, when evaluated by immunoblot, than the recombinant protein from 18×10^3 M_r (TS18) [10]. Then, it would be very interesting to express F18 protein using eukaryotic expression systems, and study the impact of the carbohydrate residues from this protein in the diagnosis properties of the molecule.

With respect to specificity of the F18 molecule, none of the sera from hydatidosis patients recognized the G-F18 protein and other recombinant proteins or peptides derived from the F18 molecule. These results are good, and in contrast with the ones obtained with the *T. solium* cyst antigen, with a very high sensitivity (100%) while the specificity was very low with respect to hydatidosis sera (7%), data which were in agreement with other works, when crude *T. solium* metacestodes antigens [33] or *T. solium* cystic fluid [46] were employed and cross-reactions with other parasite infections were detected.

Finally, it could be speculated that the high sensitivity of the F18 molecule observed with sera from patients with NCC, not in the acute stage of the disease, was related to a somatic origin for F18 antigen, which could be exhibited when the parasite is in its degeneration stage. In addition, the F18 somatic nature is also suggested taking into account that the molecule does not show hydrophobic domains, signal peptides or transmembrane regions, characteristic of secreted and/or surface antigens.

In the future, further studies will be done to elucidate the biochemical and biological properties, as well as the function of F18 protein in the *T. solium* metacestodes and in the search of new molecules to solve the diagnosis problem of patients with a single cyst, calcification cysts or with NCC forms difficult to detect by the immunodiagnostic assays.

5. Conclusions

F18 is a single copy gene from the *T. solium* metacestode, that encodes a putative polypeptide of 229 amino acids with a predicted molecular mass of 26.06×10^3 M_r. The expression of F18 cDNA in pGEX vector, besides the purification of the recombinant protein by affinity chromatography using the matrix glutathione–Sepharose™ 4B, allowed us to obtain the G-F18 antigen. Moreover, the G-F18 antigen showed good sensitivity and interesting specificity in the cysticercosis diagnosis. As a consequence, the results suggest the utility of F18 recombinant protein as an antigen for the detection and confirmation of NCC, in conjunction with clinical data and radiological findings in geographical areas where cysticercosis and hydatidosis diseases coexist.

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